Nanocrystal-Polymer Particles: Extended Delivery Carriers for Osteoarthritis Treatment

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Abstract

An efficient treatment for osteoarthritis (OA) can benefit from the local release of a high therapeutic dose over an extended period of time. Such a treatment will minimize systemic side effects and avoid the inconvenience of frequent injections. To this aim, nanocrystal–polymer particles (NPPs) are developed by combining the advantages of nanotechnology and microparticles. Nanocrystals are produced by wet milling kartogenin (KGN), which is known to promote chondrogenesis and to foster chondroprotection. A fluorescent biodegradable polymer is synthesized for intravital particle tracking. Polymer microparticles with 320 nm embedded KGN nanocrystals (KGN-NPPs) show a high drug loading of 31.5% (w/w) and an extended drug release of 62% over 3 months. In vitro, these particles do not alter mitochondrial activity in cultured human OA synoviocytes. In vivo, KGN-NPPs demonstrate higher bioactivity than a KGN solution in a murine mechanistic OA model based on histological assessment (Osteoarthritis Research Society International score), epiphyseal thickness (microcomputed tomography), OA biomarkers (e.g., vascular endothelial [...]
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1. Introduction

Osteoarthritis (OA) is the most common chronic form of articular disease\(^1\) and affects 80% of the world’s population aged 75 yr and above.\(^{[2,3]}\) OA is a leading cause of chronic disability that progressively affects cartilage, synovial membranes, subchondral bone, and periarticular tissues. The risk factors of OA include age, gender, joint biomechanics, genetic factors, and adiposity. Due to the localized nature of the disease, intraarticular (IA) drug injection\(^{[4–7]}\) is an attractive treatment approach. IA administrations may show an improved benefit–risk ratio by increasing the dose efficacy at the site of action and minimizing the risks of systemic adverse effects, drug–drug interactions, and other toxicities, specifically when the drug stays locally at the site of injection. Various formulations of hyaluronic acid and corticosteroids are approved by the Food and Drug Administration\(^{[8]}\) and currently commercially available for OA IA treatments; however, they only provide symptomatic pain relief and do not modify disease progression. Recently, kartogenin (KGN), a CBFβ-RUNX1 pathway activator, has been reported as a promising active pharmaceutical ingredient (API) for articular cartilage regeneration and protection.\(^{[9,10]}\) This small molecule is a disease-modifying OA drug (DMOAD) that is able to activate the transcription of proteins that lead to the chondrocyte differentiation of primary human mesenchymal stem cells (EC\(_{50}\) value 100 \(\times\) 10\(^{-9}\) M).\(^{[9,11]}\)

There is a high medical need to develop drug delivery systems specifically suited for the IA environment to avoid the...
rapid clearance of biologically active molecules from the joint space. To tackle this challenge, two techniques can be considered: nanocrystals and polymeric particles. On the one hand, the formulation of nanocrystals is a robust approach to control the delivery of poorly water-soluble API, such as KGN ($\log P$: 4.37). Due to their structure, nanocrystals tend to generate zero-order kinetics with no burst release and reduced dose-dependent toxicity.\(^{[12]}\) On the other hand, polymeric biocompatible micro- and nanoparticles are drug delivery systems that enable extended drug release over days to months depending on their size and the selection of an appropriate polymer. Poly(DL-lactic acid) (PLA) is one of the most attractive polymeric candidates for these systems.\(^{[13]}\) When injected into the joint, modification of the particles’ size influences their phagocytosis by immune cells, the immune and inflammatory response, and, thus, their retention in the joint. Polymeric particles with diameters ranging from 10 to 25 $\mu$m were proven to be optimal for prolonged articular retention.\(^{[14]}\) Microencapsulation by spray drying seems to be a valuable method for improving the entrapment efficiency and drug loading and for decreasing drug transfer from the particles’ core to shell compared to other commonly used preparation techniques performed in aqueous media.\(^{[15]}\)

Compared to previous reports on IA administration, the aim of the present study was to develop microparticles with a very high drug loading and extended-release properties for local and potent DMOAD treatment. To this end, an unprecedented technology combining nanocrystals and polymeric microparticles was developed. To validate the use of nanocrystal–polymer particles (NPPs) for these purposes, the different steps of our investigation were

(i) to formulate KGN nanocrystals by wet milling and to protect them against crystalline regrowth, (ii) to synthesize a fluorescent polymer for the intravital tracking of particles, (iii) to encapsulate a high payload of KGN nanocrystals into polymeric particles in the appropriate size range, (iv) to assess in vitro drug release, (v) to evaluate the in vitro cytotoxicity of the particles on cultured human OA synoviocytes, and (vi) to investigate the therapeutic activity of the particles in vivo in a murine model of OA induced by destabilization of the medial meniscus (DMM).

2. Results and Discussion

2.1. NPPs as a Novel Drug Delivery System

The KGN used in the current study was purchased as a partially crystalline powder (Figure 1a). Therefore, the first step of the formulation was to perform a recrystallization process to warrant a fully crystalline form as a starting material.\(^{[16]}\) Microencapsulation by spray drying seems to be a valuable method for improving the entrapment efficiency and drug loading and for decreasing drug transfer from the particles’ core to shell compared to other commonly used preparation techniques performed in aqueous media.\(^{[15]}\)

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Figure 1. Formulation of NPPs. Scanning electron micrographs of a) KGN from the supplier, b) KGN crystals after recrystallization, c) KGN nanocrystals after the wet milling process, d) surfaces of blank NPPs, e) core of a blank NPP, g) surfaces of KGN-NPPs, and h) core of a KGN-NPP. Scale bar = 5 $\mu$m.

f) Chemical structure of PLA-Cy7. I) Schematic representation of an NPP.
polymer was needed for the intravital tracking of particles; such a polymer was obtained by covalently linking Cyanine 7 (Cy7) to PLA (Figure 1f). This fluorescent dye was selected as it allows intravital imaging (\(\lambda_{\text{ex}} 750\) nm, \(\lambda_{\text{em}} 773\) nm). The two-step synthesis route of PLA-Cy7 is reported in Figure S1a, Supporting Information. PLA was first derivatized by coupling its carboxylic acid to a dibenzocyclooctyne-amine to then allow a reaction with Cy7-azide by click chemistry. The structure of the resulting PLA-Cy7 and its intermediates was characterized by \(^1\)H-nuclear magnetic resonance (NMR) spectrometry (Figure S1b,c, Supporting Information). PLA-Cy7 has a total degree of substitution of 54%.

KGN nanocrystals, which were previously obtained by wet milling, were suspended in a polymer solution containing PLA and PLA-Cy7 with the aim of obtaining 0.1% (w/w) dye in the final product. The NPPs of KGN (KGN-NPPs) (Figure 1i) were formulated by spray drying. Compared to an emulsification–evaporation process, the fast spray drying process prevents the API from diffusing toward the surface of the particles. No residual solvent was detected (below detection limit) in the final product by gas chromatography using the USP 467 method.

Scanning electron micrographs reveal the spherical morphology of the NPPs (Figure 1d,g). The cross-sections of the KGN-NPPs (Figure 1h) have a granular surface due to the presence of nanocrystals homogeneously distributed in the core of the particles in contrast to the smooth surface of the blank NPPs (Figure 1e). Size measurements by light scattering confirm a mean size of 13.81 \(\mu\)m in the appropriate range of 10–25 \(\mu\)m, and ultra-high performance liquid chromatography (UHPLC) reveals a very high drug loading of 31.5% (w/w) (Figure 2d).

X-ray diffraction (XRD) spectra confirm the crystalline form of KGN by the high specific peak at 5.7\(^{\circ}\) on the 2\(\theta\) scale (Figure 2a), which is observed with an increasing intensity throughout the nanocrystallization steps. For the KGN-NPPs, this peak is still present, although it is smaller due to the large proportion of amorphous polymer in the formulation. The crystalline state of KGN was also assessed by modulated differential scanning calorimetry (MDSC) thermographs (Figure 2b). At 58 \(^{\circ}\)C, the thermal event corresponds to the glass transition temperature (\(T_g\)) of the amorphous PLA present in the KGN-NPPs. Then, the endothermic event (\(T_m\)) for the KGN nanocrystals and KGN-NPPs at 190 and 198 \(^{\circ}\)C, respectively, correspond to the melting point of the API, which is slightly shifted in the case of the NPPs due to the presence of the polymer in the particles.

The cumulative release of the active ingredient was monitored over 3 months under sink conditions in a buffer maintained at pH 7.4 (Figure 2c) to avoid a pH change due to the degradation of PLA and the pKa (2.91) of KGN. A burst effect (first phase: up to 8 h) is first observed and is explained by the presence of a small amount of KGN nanocrystals on the surface and in the most peripheral areas of the particles; this explanation is in agreement with the literature.[18,19] The release profile then reveals a second phase (up to day 14), which tends to follow a zero order kinetic profile (\(r^2 = 0.99\)) due to the crystalline form of the API dispersed into the particles. Finally, a third phase occurs, with a sustained release of 22.5 \(\mu\)g kg\(^{-1}\) week\(^{-1}\) until day 90 (linear regression, \(r^2 = 0.93\)). The sufficiently high KGN release rate enables to remain above the EC\(_{50}\) in vivo during 90 d.

Since KGN was embedded as nanocrystals in a biocompatible polymeric matrix releasing its content gradually, KGN-NPPs did not affect the cell viability of cultured OA human synoviocytes at high doses (100 \(\times\) EC\(_{50}\)). This was shown by a mitochondrial activity test using human fibroblast-like synoviocytes exposed to NPPs for 24 h (Figure S2a,b, Supporting Information).

In summary, the high drug loading (Figure 2d), the prolonged release of 62% of the total drug load over 90 d, and the favorable cell viability results supported further preclinical in vivo testing; that is, IA injections in a murine OA model.
2.2. Evaluation of Therapeutic Effects in a Mechanistic Murine OA Model

To evaluate the effects of KGN-NPPs on the development and progression of OA in vivo, a murine model based on the surgical DMM was chosen because this model mimics the progression of human OA. The section of the medial meniscotibial ligament was performed on the right knee of C57BL/6 mice (n = 7 per group). One week after the surgery, the first IA injection was administered at day 0 and the second one at day 29 in the medial part of the destabilized knee. The dose of injected KGN-NPPs corresponded to 2.25 mg kg⁻¹ month⁻¹ of KGN. In this study, KGN solution (1.1 µg kg⁻¹ week⁻¹ as previously reported) and blank NPPs (without drug) were used as control formulations, and a sham condition (operation without destabilization, only injected with phosphate buffered saline (PBS)) was used as a control. Monitoring of the weights of the animals did not show any significant difference among groups several weeks after surgery, confirming the nondebilitating nature of the surgical procedure (Figure S3, Supporting Information).

Figure 3. Persistence and functional effects of KGN-NPPs (n = 7 per group). a) Representative intravital fluorescence superimposed with X-ray images of mice at day 56 (source intensity scale from 0 to 2450 pmol m⁻¹ cm⁻¹). b) Percentage of intravital fluorescence in mice after IA injection of KGN-NPPs normalized at day 0 (first injection). c) Representative hematoxylin and eosin (H&E) paraffin-embedded tissue light (left) and fluorescence (right) micrographs of KGN-NPPs on the lateral side knee of a mouse at day 56. Autofluorescence of tissues (blue), and fluorescence of NPPs (PLA-Cy7) (red). Scale bar = 50 µm. d) Percentage ratio of medial to lateral tibial epiphysis thickness at day 56. e) Multiplex enzyme-linked immunosorbent assay (ELISA) analysis of vascular endothelial growth factor (VEGF) in plasma from mice at day 56. Data represent the mean ± s.d., *P < 0.0332, **P < 0.0021, ***P < 0.0002, and ****P < 0.0001 (all groups compared to sham).
The persistence of NPPs was monitored for 2 months by semi-quantitative intravital analysis of Cy7 fluorescence. The average, total, maximum, and area of the fluorescence were the tracking parameters and were normalized to those of the first injection (Figure 3b). On the one hand, at day 29, the total signal and the area of fluorescence increased significantly due to the second injection and then decreased at day 56. On the other hand, the average and maximum signals stayed constant. Intravital fluorescence of the KGN-NPPs superimposed with the X-ray skeleton of a representative mouse (Figure 3a) confirmed the highly localized retention in the joint over a two-month period. The histology study also supported the same conclusions using light and fluorescence microscopy (Figure 3c). The NPPs were present in the articular soft tissues surrounding the knee, mainly in the lateral part, which is probably due to the rodent’s movements. This result might be explained by an increase in pressure on the median side compared with the lateral side arising from the specificity of the DMM model, in which the articular space on the median side decreases, whereas it increases on the lateral side. These fluorescence experiments performed using the DMM murine model confirm that the size of the NPPs is appropriate for extended persistence into the joints for at least 2 months.

The percentage ratio of medial to lateral tibial epiphysis thickness was measured by microcomputed tomography (micro-CT) imaging (Figure 3d). The results indicate that KGN-NPPs show a trend in protecting the epiphysis and preserving its thickness, which is probably because the bone was still covered and protected by the cartilage.\textsuperscript{[21,22]}

Quantification of OA biomarkers in mice plasma at day 56 was assessed by multiplex ELISA. Compared to the KGN solution, KGN-NPP treatment led to a more significant effect on vascular endothelial growth factor (VEGF; Figure 3e), which has a key role in chondrocyte metabolism\textsuperscript{[23]} and disease progression.

No significant differences in the expression levels of inflammatory cytokines (IL-1β and TNFα) between the OA groups were observed (Figure S4, Supporting Information). The absence of an effect on the inflammatory cascade is in agreement with the specific mode of action of KGN.\textsuperscript{[11]} The blank NPPs by themselves did not induce inflammation. However, the DMM model does not reflect physiological inflammation, only the development of a very mild secondary inflammation.

Taking into account the in vitro drug release profile, the total dose of API released over 2 months should be 2.52 mg kg\(^{-1}\). A biodistribution study of the API was conducted on the main organs (liver, lungs, spleen, kidneys, and heart) to assess the fate of KGN. No API could be detected in these organs above the limit of detection of 0.5 ppb set by the UHPLC MS/MS method. This result highlights the preferential residence of the API in the joints and shows that systemic exposure is limited. The absence of API accumulation in these organs at 2 months supports the safety of the local KGN delivery approach.

The histological findings reveal moderate OA intensity two months after the destabilization (Figure 4f–h), with a maximum Osteoarthritis Research Society International (OARSI) score of 4 out 6 (no osteophytes observed). In this study, the DMM model offers the advantages of a slow disease progression compared to other surgically induced models (e.g., anterior cruciate ligament transaction).\textsuperscript{[24]} The lateral part of the knee, protected by the lateral meniscus, was also used as an internal control. OA lesions were located on the medial half of the knee joint in almost all samples, in line with the experimental settings. OARSI scores of the medial tibia correlate well with the medial femur (\(r^2 = 0.92\)) (Figure 4h). NPPs were detected in nearly tissue sections of the blank NPP and KGN-NPP groups as assessed by fluorescence microscopy. Based on light microscopy, NPPs were located either in the extracellular space of the synovial tissue/adjacent tissue and surrounded by foamy macrophages or also found within foamy macrophages partially digested. In addition, the inflammatory infiltrate contained some giant cells demarcating or digesting NPPs. At the time of sacrifice, overall no strong tissue inflammation was seen.

To facilitate the histological analysis of the cartilage, tissue sections of the different groups were also stained with safranin-O/fast green (SOFG; Figure 4a). On control OA, blank NPP and KGN solution groups, wide areas of cartilage destruction were observed with matrix loss and surface denudation compared with the sham or KGN-NPP-treated groups. No bone erosion or inflammation was seen in any of the investigated conditions using a semi-quantitative scoring. The DMM OA model induced significant OA damage as shown by the significant differences in the OARSI scores\textsuperscript{[25]} of the medial tibia, medial femur, medial cleft/cartilage erosion, and medial cartilage thinning when compared with the corresponding lateral sides and untreated animals (Figure 4f–h). For all these scores, a significant improvement was observed with the use of KGN-NPPs compared to KGN solution. Cleft/cartilage erosion and cartilage thinning/loss scoring (from 0 to 3) revealed the bioactivity of KGN-NPPs on the medial part (Figure 4f,g). The high API dose provided by the KGN-NPPs enabled cartilage protection and regeneration, maintaining an effective drug concentration despite joint clearance. By contrast, as shown in Figure 4 and in the study of Johnson et al.,\textsuperscript{[9]} KGN solution requires weekly injections to be effective, which would be a clinically inadequate administration scheme/frequency.

Furthermore, immunohistochemistry (IHC) tests were also conducted to qualitatively evaluate the presence of four other biomarkers (collagen type 2 (Col2), Aggrecan, matrix metallopeptidase 13 (MMP13), and a disintegrin and metalloproteinase with thrombospondin motifs 5 (Adams5)) in the medial tibial cartilage that are involved in cartilage remodeling and OA pathogenesis (Figure 4b–e). Indeed, Col2 and Aggrecan are two main components of the extracellular matrix of cartilage that provide tensile strength and compressive resilience, respectively. MMP13 is a collagenase involved in collagen degradation, and Adams5 is one of the most active proenzymes involved in Aggrecan cleavage. The staining levels of Col2 and Aggrecan in chondrocytes were higher in tissue sections from healthy cartilage and groups treated with KGN-NPPs (Figure 4b,c) than in OA and blank NPP groups. In addition, MMP13 (Figure 4d), a collagenase with substrate specificity that targets collagen for degradation, was not inhibited by KGN, which is in line with the previous report.\textsuperscript{[9]} Adams5 was less expressed in the KGN-NPP group compared with the sham and KGN solution groups...
This finding is in line with previous data\cite{10} showing that KGN indirectly decreases Adamts5 expression. However, Adamst5 was not present in the medial tibial cartilage of the OA and blank NPP groups. This result is probably due to the large extent of cartilage degradation in these mice. Consistent with his hypothesis, Adamts5 was readily detectable in the lateral tibial cartilage of these mice (Figure S4, Supporting Information). Finally, taking together these data, the therapeutic effect of KGN, a relevant DMOAD, was significantly improved using the new NPP delivery system on a DMM murine model.

(Figure 4e). This finding is in line with previous data\cite{10} showing that KGN indirectly decreases Adamts5 expression. However, Adamst5 was not present in the medial tibial cartilage of the OA and blank NPP groups. This result is probably due to the large extent of cartilage degradation in these mice. Consistent
3. Conclusion

In this proof-of-concept study, NPPs were successfully designed to provide controlled, extended release of an API from biocompatible, biodegradable polymeric particles containing significant amounts of API nanocrystals. Their bioactivity was investigated in a disease-relevant OA mouse model. KGN-NPPs appeared to have a protective effect on the cartilage and epithysis of the medial tibia and to significantly reduce VEGF and Adami'ts5 expression. KGN-NPPs can achieve more efficient joint protection than KGN solution, which is eliminated rapidly by IA clearance.

The development of treatment that avoids systemic side effects and reduces the number of IA injections fulfills an unmet medical need and is a real challenge for personalized medicine. NPPs, as innovative formulations that take advantage of nanotechnology and microparticles, show promise as a safe drug delivery system to locally treat diseases over an extended period of time with a high drug dose. For the first time nanocrystals have been embedded in biodegradable microparticles. This approach avoids the potential irritating effect of pure nanocrystals directly injected into joint spaces. As the next step toward an effective treatment of chronic diseases such as OA, this approach should be tested in larger animals, such as rabbit and horses, for pharmacokinetics, safety, and efficacy. Furthermore, the concept of NPPs could be expanded to deliver a variety of medications, targeting diseases that require sustained and controlled drug release.

4. Experimental Section

Formation of KGN Nanocrystals: Five milligrams of KGN purchased from Merck Millipore (Billenca, MA, USA) were dissolved in 300 µL of tetrahydrofuran directly into a 2.0 mL tube with 579 mg of 0.5 mm zirconium TriplePure M-Bio Grade beads purchased from Sigma-Aldrich (St. Louis, MO, USA). Crystallization was performed by adding 600 µL of distilled water as nonsolvent, and the tube was left open overnight at 4 °C to allow slow evaporation of the solvent. Then, 100 µL of TPGS (4% (w/v)), purchased from Sigma-Aldrich, were added as a stabilizer. Size reduction of the crystals was performed by placing the tubes in a cold mixture of MeOH/ EtO2 1:1 and centrifugation (10 min, 3000 × g) were necessary to clarify the polymer. The final precipitate was dried under vacuum to give 336 mg (0.0246 mmol, 84%) of PLACy7 as a green solid. 1H-NMR spectra were acquired on a Varian Anova Gemini 300 MHz spectrometer (Palo Alto, CA, USA). NMR spectra were processed with Mestra version 9.0.1 software package. 1H-NMR (600 MHz, DMSO-d6), δ (ppm): 1.35–1.55 (m, 549H), 2.51 (s, 1.25H), 3.58 (s, 1.25H), 4.11 (t, 0.89H), 5.10–5.25 (m, 180H), 5.51 (d, 0.91H), 6.11–6.17 (m, 1.38), 7.16–7.98 (m, 8.66H).

Formulation of NPPs: PLA (Purac Mw 65 kDa) was donated by Corbion (Diemen, the Netherlands). Approximately 38 mg mL−1 of PLA and 1.7 mg mL−1 of PLACy7 were dissolved in dichloromethane. Then, KGN nanocrystals were suspended in the polymer solution at a concentration of 23.4 mg mL−1 and were spray dried to formulate KGN-NPPs containing Procept 4M8-Trix protected from the light with a nitrogen closed loop recirculation unit (Procept Processing Equipment, Zelzate, Belgium). The following processing parameters were used: M cyclone size; 20% pump position; small (Ø 1.6 mm) tubing size for the pump; 80 °C temperature inlet; 0.4 m3 min−1 air flow; 0.6 mm nozzle size; 5 L min−1 nozzle size flow; 40–50 mbar differential pressure cyclone; and 140 L min−1 air carrier flow. KGN-NPPs were collected, dried in an oven at 37 °C under vacuum, lyophilized and stored at 4 °C protected from light. Blank NPPs were formulated with the same method except that the mass of nanocrystals was substituted with PLA.

Size, Morphology, Encapsulation Efficiency, and Drug Loading: The size of the NPPs was determined by laser light diffraction (Mastersizer S, Malvern Instruments Ltd., Malvern, UK). DLS (Nanosizer, Malvern, England) was used to determine the size of the nanocrystals and the zeta potential of NPPs. The morphology was determined by scanning electron microscopy (Jeol Microscope, JSM-7001TA, Tokyo, Japan) at an accelerating voltage of 5 kV. Cross sections were obtained by first embedding NPPs in an epoxy resin, then cutting them on a Ultramicrotome Leica UCT (Wetzlar, Germany) using a diamond blade. The drug encapsulation efficiency (Equation (1)) and drug loading (Equation (2)) into the microparticles were determined by reversed-phase UHPLC and were calculated based on the following equations:

\[ \text{Encapsulation efficiency} = \frac{\text{wt drug entrapped}}{\text{wt theoretical drug loading}} \times 100 \quad (1) \]

\[ \text{Drug loading} = \frac{\text{wt drug entrapped}}{\text{wt microparticles}} \times 100 \quad (2) \]

UHPLC: KGN was quantified by reversed-phase UHPLC using a Thermo Fisher Accela system and a C18 Hypersil gold column 50/2.1 with a 1.9 µm bead particle size (Thermo Scientific, Waltham, MA, USA). The mobile phase comprised 0.1% v/v trifluoroacetic acid (TFA) in water.
or injected with PBS. On day 56, the total blood was collected for further analysis via intracardiac puncture under deep anesthesia, and the mice were sacrificed by spinal cord dislocation. Blood was added to tubes containing 1 mg of ethylenediaminetetraacetic acid dipotassium salt purchased from Sigma-Aldrich and was directly centrifuged at 1000 × g for 15 min and 10 000 × g for 10 min at 4 °C. Plasma was collected and stored at −80 °C. Heart, kidneys, liver, spleens, and lungs were harvested and rinsed with NaCl 0.9% and were frozen at −20 °C.

Histology and Evaluation: The knee joints were collected and fixed in 4% paraformaldehyde for 24 h. Whole joints were decalcified in 10% (w/v) Tris-TFA for 5 weeks on a shaker. The joint was embedded in paraffin, and 5 μm frontal sections were taken at two levels of the joint at 800 μm intervals. Slides were stained with H&E, toluidine blue, and SOFG. The severity of OA was assessed and scored in a blinded manner. Cartilage thinning and cleft/cartilage erosion, bone erosion, and joint inflammation were evaluated using the following semi-quantitative scoring system: 0 (no signal), 1 (mild), 2 (moderate), 3 (severe). Furthermore, the overall osteoarthritic damage was evaluated using the published and recommended OARSI score[25] from 0 (normal) to 6 (very severe) as follows: 0 (normal), 0.5 (loss of Safranin-O without structural changes), 1 (small fibrillations without loss of cartilage), 2 (vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina), 3 (vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface), 4 (vertical clefts/erosion to the calcified cartilage extending to 25%–50% of the articular surface), 5 (vertical clefts/erosion to the calcified cartilage extending to 50%–75% of the articular surface), and 6 (vertical clefts/erosion to the calcified cartilage extending >75% of the articular surface). Fluorescence histology was also performed to analyze NPP distribution into the joints. Unstained paraffin-embedded joint tissue sections were analyzed by fluorescence microscopy (filters: DAPI and Cy7) on a Zeiss Axioscan.Z1 scanner (Carl Zeiss, Jena, Germany).

IHCl: Serial sections adjacent to those assessed by conventional histology were dewaxed in Neo-Clear (Merck Millipore, Darmstadt, Germany) and were rehydrated at 5 min per step through a graded series of alcohols, distilled water, wash buffer, and PBS. Antigen retrieval was accomplished in 10 × 10 −3 μM citrate at pH 6 at 80 °C for 20 min. Functionally distinct zones around tissues sections were delimited with PAP Pen (Agilent Technologies). Blocking endogenous peroxidase (Agilent Technologies) was added for 5 min, washed 2–5 min with wash buffer, quenched for 1 h in blocking buffer (5% goat serum and 3% Triton X-100 in PBS), and washed 2–5 min again with wash buffer. Anti-Col2 (ab85266), anti-Aggreca (ab140707), anti-MMP13 (ab39012), and anti-Adamts5 (ab182795) (Abcam, Cambridge, UK) were the primary antibodies used at 1/100 dilution in antibody diluent (Agilent Technologies). Sections were incubated in a humidified chamber at 4 °C overnight and washed with wash buffer for 20 min on the second day. EnVision® System (Agilent Technologies), which is based on a horseradish peroxidase-labeled polymer conjugated to the secondary antibodies, was added for 30 min, followed by 2–5 min in wash buffer. Peroxidase activity was revealed by immersion in DAB substrate (Agilent Technologies). The sections were immersed for 3 s in hemalum/water (1:1) and then placed in hot water. Finally, the sections were dehydrated, covered with a coverslip and scanned by a Zeiss Axioscan.Z1 scanner (Carl Zeiss, Jena, Germany).

Biodistribution Study: Lungs, spleen, kidneys, heart, and liver from sacrificed mice were ground for 60 s in 2 mL tubes containing 200 μL of acetonitrile and 6 metal beads (1/8") using a FASTPREP-24 Instrument (MP Biomedicals, CA, Santa Ana, CA, USA). Quantitative analysis of KGN was performed using a Waters Acquity UPLC I-class System (Waters, Milford, MA, USA) equipped with a binary solvent manager delivery pump, a sample manager autosampler with flow through a needle (SM-FTN). The chromatographic system was coupled to a Waters Xevo TQ-S triple quadrupole. Chromatographic separation was performed on a C18 Hypersil gold column (50/2.1, 1.9 μm bead particle size, Thermo Scientific). The mobile phase consisted of 0.1% formic acid water (A) and ACN (B). The optimized UHPLC elution conditions were:

(A) and 0.1% v/v TFA in acetonitrile (B), and the following gradient elution sequence was applied at a flow rate of 400 μL min −1: 30%–95% A (0–3 min), 95%–10% A (3–4 min), 10%–30% A (4–4.5 min), and 30% A (4.5–5 min) at a flow rate of 400 μL min −1.
were 30%–95% A (0–4 min) and 95%–30% A (4–5 min) at a flow rate of 400 \( \mu \text{L min}^{-1} \) with an injection volume equal to 10 \( \mu \text{L} \). The Xevo TQ-S detector was exclusively operating in negative ESI mode. Optimal parameters for a high-sensitivity quantitative method were capillary 3 kV, cone 30°, extractor 3 V, span 300, gain 100, and collision 20. Data processing and peak integration were acquired with TargetLynx software.

**Micro-CT:** Two months after the first IA injection, the mice were sacrificed, and their right knee joints were scanned on the same day using micro-CT (Quantum FX, PerkinElmer, Hopkinton, MA, USA) with a voxel size of 10 \( \mu \text{m} \). The X-ray tube voltage was 90 kV, and the current was 88 \( \mu \text{A} \). The software OsiriX Lite (OsiriX Foundation, Geneva, Switzerland) was used to measure the epiphyseal thickness from the articular cartilage surface to epiphyseal line at the center of the medial and lateral tibial epiphysis for all samples. All the samples were analyzed with a window level of 1500 and a window width of 3000.

**Intravital Fluorescence:** Intravital fluorescence based on the Cy7 emission spectrum in mice was assessed weekly using a Maestro M1 imaging system (Perkin Elmer, Cambridge Research and Instrumentation Inc., Massachusetts). Fluorescence area, maximum, total, and average signals were normalized to the initial \((t_0)\) area. 3D coregistration of micro-CT and KGN-NPPs fluorescence was performed at 2 months. The 3D reconstructed fluorescence was based on the Cy7 emission spectrum using the IVIS Spectrum In Vivo Imaging System (Xenogen, PerkinElmer, Waltham, MA, USA).

**Multiplex ELISA:** Plasma samples from the mice were analyzed using a multiplex kit and beads purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), according to the specifications of the manufacturer on the following OA biomarkers: IL-1\( \beta \), TNF\( \alpha \), and VEGF. Standard curves for each OA biomarker were generated using the reference cytokine concentrations supplied by the manufacturers. The raw data were analyzed using Bio-Plex Manager software 6.1 to obtain concentrations in \( \mu \text{g ml}^{-1} \).

**Statistical Analysis:** Data are expressed as mean \pm standard deviation (s.d.). The statistical significance of the results was determined using Student’s t-test (two groups), one-way or two-way analysis of variance (ANOVA, multiple groups), and a multiple comparison function (Tukey) in Prism 7.2 (GraphPad) with an alpha level of 0.05. \( P \) values correspond to \(*P < 0.0332\), \(*\*P < 0.0021\), \(*\*\*P < 0.0002\), and \(*\*\*\*P < 0.0001\).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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Note: Poly(DL-lactide) (PLA) has been corrected to Poly(DL-lactic acid) on 22 Feb 2018 after original online publication.

**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

biodegradable microparticles, intra-articular administration, kartogenin, nanocrystals, osteoarthritis

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